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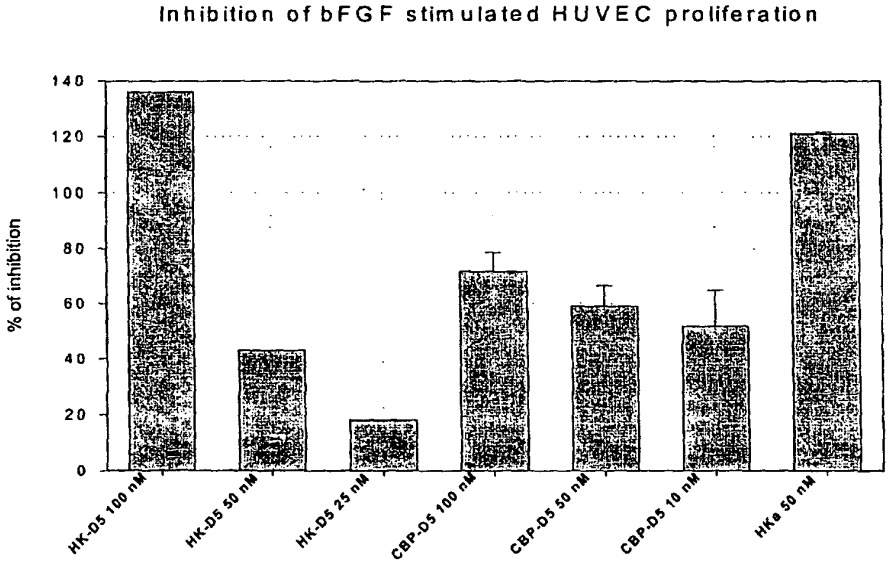
(54) Title: HUMAN KININOGEN D5 DOMAIN POLYPEPTIDES



(57) Abstract: Peptides form the human kininogen D5 domain and fusion peptides thereof having angiogenesis-inhibitory activity. These peptides are used in diagnosis and therapy of diseases associated with endothelial cell migration and proliferatrtion, e.g., the treatment of cancer. The invention further relates to nucleic acid molecules encoding said peptides, antibodies to said peptides and methods for isolating said peptides and cells expressing them.

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HUMAN KININOGEN D5 DOMAIN POLYPEPTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the field of biochemistry and medicine relates to angiogenesis-inhibitory peptides and polypeptides comprising parts of the D5 domain of human kininogen and their use in diagnosis and therapy of diseases associated with endothelial cell migration and proliferation. In particular these polypeptides are useful in treating subjects with cancer.

Description of the Background Art

In adults, new blood vessels are formed through the process of angiogenesis, in which new capillaries sprout from the existing vasculature (Risau W., *Nature 386*:671-674, 1997). The endothelium of a non-angiogenic vessel is normally quiescent, whereas angiogenic endothelial cells ("ECs") proliferate actively. Angiogenesis is a complex process that involves (1) degradation of the underlying basement membrane by angiogenic ECs, (2) loss of EC adhesion, (3) -migration and proliferation of the detached cells toward the angiogenic stimulus, and (4) ordered reassociation of these cells to form a new vessel. Angiogenesis in the adult occurs only in "pathological" situations, such as in response to wound healing, tissue ischemia or neoplasia.

The most thoroughly studied angiogenic factors in tumors are basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial cell growth factor (VEGF) ((Pepper MS *et al.*, *Curr. Topics Microbiol. Immunol.* 213 (Pt 2):31-67, 1996). These proteins are produced by neoplastic cells or their stroma, often in response to tissue hypoxia, and promote EC migration and proliferation through interactions with specific cell surface receptors with intrinsic tyrosine kinase activity (Thomas KA., *J Biol Chem 271*:603-606, 1996). The importance of each of these factors in the neovascularization and growth of tumors has been demonstrated in animal models through the use of agents which either bind and inactivate these growth factors or their cell-surface receptors ((Lin P *et al.*, *Cell Growth Diff. 9*:49-58, 1997; Wang Y. *et al.*, *Nature Med 3*:887-893, 1997; Skobe M *et al.*, *Nature Med 3*:1222-1227, 1997).

Angiogenesis is thought to be regulated under certain conditions by cryptic polypeptides released from larger proteins by proteolysis within the tumor milieu. It is becoming apparent that proteolytic fragments of plasma proteins or extracellular matrix (ECM) proteins may play an important role (Hanahan D *et al.*, *Cell 86*:353-364, 1996). To date, several polypeptides with such activities have been identified: angiostatin, endostatin, PEX (the C-terminal hemopexin

domain of matrix metalloprotease 2 (MMP-2) (Brooks PC *et al.*, *Cell* 92:391-400, 1998), the N-terminal 16 kD fragment of prolactin and a 29 kDa fragment of fibronectin (O'Reilly MS *et al.*, *Cell 79*:315-328, 1994; Folkman, J., *Sci. Amer. 230*:150-154, 1996. Both intact thrombospondin 1 (TSP-1) as well as peptides derived from its procollagen and properdin-like type 1 repeats are anti-angiogenic (Good DJ *et al.*, *Proc. Natl. Acad. Sci. USA 87*:6624-6628, 1990). The ability of several of these fragments to inhibit tumor growth or induce tumor regression and/or dormancy in preclinical models has led to enthusiasm for the potential use of angiogenesis inhibitors for therapy of human neoplasms.

The prototype inhibitor is angiostatin which consists of kringles 1-4 of plasminogen and inhibits the growth of experimental tumors in mice (O'Reilly *et al.*, *supra*). Angiostatin may be generated by serine proteases, as well as matrix metalloproteinases 7 and 9 and macrophage metalloelastase. A second anti-angiogenic polypeptide, endostatin, is a 20 kDa C-terminal fragment of the basement membrane protein, collagen XVIII. Endostatin also inhibits tumor growth (Folkman, *supra*), and its efficacy in repeatedly inducing tumor regression in animals in which tumors are allowed to regrow between treatment cycles suggests that development of resistance to this agent is unlikely. Repeated cycles of therapy with endostatin have also induced long-term tumor dormancy. A third anti-angiogenic protein fragment, PEX (Brooks *et al.*, *supra*). Association of MMP-2 with the integrin $\alpha_v\beta_3$, which is selectively expressed on angiogenic endothelial cells, promotes cell surface collagenolytic activity and cellular invasiveness. Autocatalysis of MMP-2 leads to the release of PEX, which continues to bind $\alpha_v\beta_3$ through a non-RGD-dependent mechanism, thereby competing with its parent molecule for binding to the integrin, and impairing cellular collagenolytic activity. Elevated levels of PEX occur in tumor tissue, and recombinant PEX inhibits tumor growth (Brooks *et al.*, *supra*).

Biochemistry of high molecular weight kininogen

Two forms of kininogen, high molecular weight kininogen (HK, M_r=120kDa) (Figure 1), and low molecular weight kininogen (LK, M_r = 68 kDa), have been identified in human plasma (Jacobsen S *et al.*, *Br J Pharm 29*:25-36, 1967). HK is an α-globulin with a plasma concentration of 90 μg/ml (Proud D *et al.*, *J Lab Clin Med 95*:563-5574, 1980) (Figure 1), and LK is a α-globulin with a plasma concentration of 220 μg/ml (Muller-Esterl W *et al.*, *Biochim Biophys Acta 106*:145-152, 1982). These proteins are derived from the alternative splicing of a single gene (Kitamura N *et al.*, *J Biol Chem 260*:8610-8617, 1985), and share a common heavy (H) chain, which contains domains 1, 2 and 3, termed D1, D2 and D3 (Colman RW *et al.*, *Blood*

90:3819-3843, 1997). However, while LK contains only a 4 kDa light (L) chain ($D4_L$), the ~46kDa L chain of HK contains domains 5 and 6 (D5 and D6, respectively).

Each domain of HK has a unique function. For example, D1 binds calcium, and D2 inhibits calpain (Colman *et al., supra).* The cell binding regions of HK are contained within D3 and D5, while D6 binds plasma prekallikrein and coagulation Factor XI. D6 remains free following the binding of HK to cells, indicating that it may serve as an "acquired receptor" for Factor XI or kallikrein.

In intact HK, D4 links the H and L chains; D4 also includes the nonapeptide, bradykinin which is released from HK by kallikrein via cleavage between Lys_{362}-Arg_{363} and Arg_{371}-Ser_{372}, leaving behind a cleaved molecule consisting of a 62 kDa H chain and 56-62 kDa L chain, which are bonded by an intrachain disulfide between Cys_{10} and Cys_{596}. A subsequent cleavage at a site near the N-terminus of D5, results in reduction of the M_r of the L chain to ~45 kDa (Kaplan AP *et al., Blood 70*:1-15, 1987). Released BRADYKININ is a potent vasodilator and an agonist for ECs. Kallikrein-mediated cleavage of HK occurs on the EC surface, and may be mediated (a) directly by plasma kallikrein or (b) after binding of prekallikrein to cell-bound HK, followed by its activation to kallikrein by an EC cysteine protease. Thus the EC is an important site for HK_a generation. Phorbol myristoyl acetate (PMA)-stimulated ECs bind increased amounts of HK (Colman *et al., supra*) suggesting acceleration of this process on "activated" ECs. The observation that ECs produce HK mRNA and protein further supports the physiological importance of this process (Schmaier AH *et al., J Biol Chem 263*:16327-16333, 1988).

The release of BRADYKININ from HK is accompanied by a structural rearrangement in the remaining two-chain kininogen molecule, HK_a and the acquisition of several novel properties. For example, cleavage of HK to HK_a allows the latter to bind to artificial anionic surfaces (Colman *et al., supra*); interactions that are mediated by residues of the His-Gly-rich region within D5 of HK_a (amino acids 420-458) (DeLa Cadena RA *et al., Protein Sci 1*:151-160, 1992; Kunapuli SP *et al., J Biol Chem 268*:2486-2492, 1993).

Furthermore, HK_a, but not HK, is anti-adhesive, inhibiting the spreading of osteosarcoma and melanoma cells on vitronectin, and of ECs, platelets and mononuclear cells on vitronectin and fibrinogen (Asakura S *et al., J Cell Biol 116*:465-476, 1992). Rotary shadowing electron microscopy demonstrated that the structural rearrangement of HK_a involves a change in the orientation of HK_a domains relative to each other.

HK exists as a linear array of three linked globular regions, with the two peripheral regions connected by a thin strand (Colman RW *et al.*, *J Clin Invest 100*:1481-1487, 1997). The strand may represent the disulfide bridge between D1 and D6, as it is no longer apparent following reduction. Studies with epitope-specific monoclonal antibodies (mAbs) determined that the globular domains on the ends of HK represent the prekallikrein-binding region (within D6 of the L chain) and the cysteine protease inhibitor region (D2 and D3 of the H chain), while the central nodule represents the anionic surface binding region within D5.

After kallikrein-mediated cleavage, the two-chain molecule, HK_a, retains the trinodular structure, though the three globular regions rearrange in a pattern resembling vertices of a triangle. In this structure, the anionic surface binding and prekallikrein binding regions are more closely apposed. Because the EC binding regions within HK have been mapped to sites within D3 of the H chain and D5 of the L chain ((Reddigari SR *et al.*, *Blood 81*:1306-1311, 1993; Herwald H *et al.*, *J Biol Chem 270*:14634-14642, 1995; Hasan AAK *et al.*, *J Biol Chem 269*:31822-31830, 1994; Hasan AAK *et al.*, *J Mol Biol 219*:717-725, 1995) and since the latter regions in the linear sequence overlap extensively with the anionic surface binding regions of HK_a, the orientation of the cellular binding regions within HK and HK_a must differ. This conclusion implies that HK and HK_a are likely to interact differently with ECs, a hypothesis supported by functional studies demonstrating that HK_a, but not HK, is a potent inhibitor of proliferation and inducer of apoptosis in ECs.

Interactions of HK with ECs

A. Identification of cell binding regions within HK

Several reports indicate that HK binds with high affinity to human umbilical vein ECs (HUVEC) (Reddigari *et al.*, *supra*; van Iwaarden F *et al.*, *J Biol Chem 263*:4698-4703, 1988; Zini JM *et al.*, *Blood 81*:2936-2946, 1993; Hasan AAK *et al.*, *Blood 85*:3134-3143, 1995). The presence of Zn^{2+} is an absolute requirement for binding, whereas Ca^{2+} either inhibited or had no effect on binding. Internalization of HK has also been reported (van Iwaarden F *et al.*, *Blood 71*:1268-1276, 1988).

The binding of HK to ECs is mediated through interactions involving both its H and L chains, and several studies have led to the identification of specific regions that mediate binding within D3 (Herwald H *et al.*, *supra*) and D5 (Hasan AAK *et al.*, J Mol Biol, *supra*) (one of which overlaps with BRADYKININ within D4). These regions were identified by the ability of

synthetic peptides with corresponding sequences to compete with intact, labeled HK for binding to HUVEC.

In contrast to HK, little information is available concerning the binding of HK_a to ECs. In one study, cleavage of biotinylated HK by increasing amounts of kallikrein led to a progressive diminution in binding of the cleaved ligand. In contrast, others reported that HK_a was more potent than unlabeled HK in inhibiting the binding of radiolabeled HK to ECs (IC_{50} = 73 nM for HK_a vs 335 nM for HK) (Reddigari *et al.*, *supra*). Although these IC_{50} values are difficult to reconcile with a reported K_d (30-40 nM) for the binding of HK to ECs, they nevertheless suggest differences between HK and HK_a in their interactions with cells.

B. <u>Endothelial cell HK/HK_a receptors</u>

HK_a inhibition of EC proliferation *in vitro* is a unique property of HK_a as HK, which binds to ECs, nevertheless lacks this antiproliferative effect. Moreover, the observed difference in binding to ECs exhibited by HK and HK_a suggests potential differences in function. HK_a could inhibit EC proliferation by several mechanisms. First, it might induce detachment of ECs from their matrix through direct interactions with integrins, thereby leading to interruption of integrin-mediated signaling and MAP kinase phosphorylation, leading to apoptosis. However, other than one report that single-chain HK binds to Mac-1 ($\alpha_M\beta_2$ or CD11b/CD18) on monocytes, there is no evidence for interactions of kininogen with integrins.

The binding of HK_a to ECs was also not inhibited by a blocking antibody against the β_3 integrin chain, suggesting that HK_a does not interact with $\alpha_v\beta_3$, an integrin which plays an important role in angiogenesis (Colman RW *et al, J Clin Invest 100*:1481-1487, 1997). HK_a might interact in either a specific or non-specific manner with an ECM protein(s), thereby preventing its interaction with an EC integrin receptor. However, there is no data to support this hypothesis. The fact that HK_a inhibited the proliferation of HUVEC plated on fibronectin, gelatin, and Matrigel, suggested effects independent of matrix identity. HK_a might inhibit the binding of growth factors to cellular glycosaminoglycans, such as heparan sulfate, or to specific growth factor receptors. However, this explanation is unlikely, since withdrawal of growth factors does not lead to EC apoptosis within 6 hours--a time frame in which HK_a induced apoptotic changes.

McCrae's group recently observed that the cleaved form of human HK_a inhibited bFGF-stimulated angiogenesis *in vivo*. (Zhang J-C *et al.*, *FASEB J.* 14:2589-600, 2000). *In vitro*, HK_a potently inhibited the proliferation of HUVEC and human dermal microvascular ECs

(HDMVEC), inducing EC apoptosis. Several peptides were identified with sequences corresponding to the binding regions within D3 and D5 of HK_a that inhibited EC proliferation at low μM to nM concentrations. Comparison of the sequences of overlapping peptides used in these studies led to the identification peptides of 4-8 amino acids that mediated this activity. Compared to the antiproliferative effects, the anti-adhesive effects of HK_a appear to be of less importance since EC adhesion was only modestly inhibited at HK_a concentrations > 100 nM, whereas anti-proliferative effects were observed at concentrations as low as ~ 1 nM.

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SUMMARY OF THE INVENTION

The present invention is directed to a polypeptide that corresponds to the D5 domain of human kininogen, or a biologically active peptide fragment, homologue or other functional derivative thereof which is has one or more of the following properties:

(a) inhibits angiogenesis at a IC_{50} of at least about 1 μM;

(b) binds to a D5 binding site on an endothelial cell with an affinity characterized by a K_d of about 1μM or lower as measured in a direct binding assay to activated endothelial cells or in a competitive binding assay to purified D5 receptor;

(c) activates one or more signaling pathways leading to induction of apoptosis in an endothelial cell; or

(d) inhibits a signaling pathway required for maintenance of endothelial cell viability.

The D5 domain preferably has the amino acid sequence SEQ ID NO:2.

In another embodiment, the above polypeptide or peptide fragment has between about 8 and about 32 or between about 16 and about 32 amino acids which includes, or consists essentially, of one or more repeats of a sequence selected from the group consisting of:

(a) GHKFKLDDDLEHQGGH (SEQ ID NO:4);

(b) KHGHGHGKHKNKGKKN (SEQ ID NO:5);

(c) HKNKGKKNGKHNGWKT (SEQ ID NO:6); and

(d) HKNKGKKN (SEQ ID NO:7).

Another The polypeptide or peptide fragment as above has between about 4 and about 16 amino acids and includes one or more repeats of a sequence **HKXK** (SEQ ID NO:8) where X is a neutral or aromatic amino acid.

The present invention also provides a D5 fusion polypeptide having a first fusion partner comprising all or a part of the D5 domain polypeptide, or a peptide fragment, homologue or other functional derivative of the D5 polypeptide, which (i) is fused directly to a second polypeptide or, (ii) optionally, is fused to a linker peptide sequence that is fused to the second polypeptide, which fusion polypeptide has one or more of the following properties:

(a) inhibits angiogenesis at a IC_{50} of at least about 1 μM;

(b) binds to a D5 binding site on an endothelial cell with an affinity characterized by a K_d of about 1μM or lower as measured in a direct binding assay to activated endothelial cells or in a competitive binding assay to purified D5 receptor;

(c) activates one or more signaling pathways leading to induction of apoptosis in an endothelial cell; or

(d) inhibits a signaling pathway required for maintenance of endothelial cell viability.

The D5 fusion polypeptide may comprise any of the above polypeptides, peptide fragments, homologues or other functional derivatives, fused to a second polypeptide. Preferably, the binding partner molecule is a protein or peptide that increases the expression, stability or biologic or pharmacologic activity of the fusion polypeptide when compared to the D5 polypeptide, fragment homologue or derivative alone. A preferred binding partner molecule is thioredoxin, calmodulin binding protein, maltose-binding protein or glutathione-S-transferase.

In the fusion polypeptide above, the second polypeptide may be one or more domains of an Ig heavy chain constant region, preferably having an amino acid sequence corresponding to the hinge, C_H2 and C_H3 regions of a human immunoglobulin Cγ1 chain.

The above fusion polypeptide may comprise a linear multimer of two or more repeats of monomers of the first fusion partner linked end to end, directly or with a linker sequences present between the monomer repeats. An example is a dimeric or trimeric fusion polypeptide which is a tandemly linked dimer or trimer of the above fusion polypeptide.

The linker in the above fusion is preferably cleavable by an enzyme that is present and active in the vicinity of, or in cells of, a tumor, such that the first fusion partner is released from the fusion polypeptide when the enzyme acts on the fusion polypeptide. Preferred enzymes are a matrix metalloprotease, urokinase, a cathepsin, plasmin or thrombin, which act to release D5 *in*

vivo (or *in situ*) in the tumor milieu. A preferred linker is a peptide having the sequence VPRGSD (SEQ ID NO:9) or DDKDWH {SEQ ID NO:10).

In another embodiment, this invention is directed to an isolated nucleic acid molecule that encodes any of the above polypeptides, fragments, homologues or other functional derivatives. A preferred nucleic acid has the sequence SEQ ID NO:3. Another preferred nucleic acid molecule encodes a fusion polypeptide as above. For example, the nucleic acid molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide that is all or a part of a D5 domain polypeptide SEQ ID NO:2 or encodes a homologue or other functional derivative thereof;

(b) optionally, fused in frame with the first nucleic acid sequence, a linker nucleic acid sequence encoding a linker peptide; and

(c) a second nucleic acid sequence that is linked in frame to the first nucleic acid sequence or to the linker nucleic acid sequence and that encodes a second polypeptide.

Also included is an isolated nucleic acid molecule that hybridizes with any of the above nucleic acid molecules under stringent conditions..

The above polypeptide or a biologically active fragment, homologue or other functional derivative may be produced by recombinant expression of the above nucleic acid molecules.

Also provided is an expression vector comprising a nucleic acid encoding any of the above polypeptides or functional derivatives, operatively linked to

(a) a promoter and

(b) optionally, additional regulatory sequences that regulate expression of the nucleic acid in a eukaryotic cell.

The expression vector preferably comprises the above nucleic acids operatively linked to (a) a promoter and (b) optionally, additional regulatory sequences that regulate expression of the nucleic acid in a eukaryotic cell.

Preferred expression vectors are plasmids or viral vectors.

Also provided is a cell transformed or transfected with any of the above nucleic acid molecules or expression vectors. Preferred cells are mammalian cell, most preferably, human.

Also provided is an isolated mammalian tumor cell transfected with an exogenous nucleic acid molecule encoding a mammalian D5 polypeptide or a biologically active fragment, homologue or other functional derivative thereof, such that when the protein, fragment,

homologue or derivative is expressed by or secreted from the tumor cell, and the tumor cell is contacted with an endothelial cell, the tumor cell or the secreted product

(a) binds to the endothelial cell; or

(b) activates one or more signaling pathways leading to induction of apoptosis in the endothelial cell; or

(c) inhibits a signaling pathway required for maintenance of endothelial cell viability.

The present invention is directed to an antibody that is specific for an epitope of a human kininogen D5 domain polypeptide, preferably a linear or conformational epitope of the polypeptide having SEQ ID NO:2. The antibody may be specific for an epitope present in a peptide selected from the group consisting of

(a) GHKFKLDDDLEHQGGH (SEQ ID NO:4);

(b) KHGHGHGKHKNKGKKN (SEQ ID NO:5);

(c) HKNKGKKNGKHNGWKT (SEQ ID NO:6); and

(d) HKNKGKKN (SEQ ID NO:7)

Preferred antibodies are monoclonal, more preferably, a human or humanized monoclonal antibody. The foregoing antibody is preferably one which, upon administration to a subject with a tumor, inhibits tumor growth or angiogenesis.

Also provided herein is an angiogenic endothelial cell-targeting pharmaceutical composition, preferably in a form suitable for injection comprising, (a) the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative; and (b) a pharmaceutically acceptable carrier.

In another embodiment, an angiogenic endothelial cell-targeting-targeting therapeutic composition comprises (a) an effective amount of the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative of any of claims bound directly or indirectly to a therapeutically active moiety, such as a radionuclide; and (b) a therapeutically acceptable carrier. The radionuclide may be ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{217}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, or ^{109}Pd.

The invention provides a method for inhibiting endothelial cell migration, proliferation, invasion, or angiogenesis, or for inducing endothelial cell apoptosis, comprising contacting endothelial cells involved in undesired migration, proliferation, invasion, or angiogenesis with an effective amount of the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative.

Also provided is a method for treating a subject having a disease or condition associated with undesired endothelial cell migration, proliferation, invasion or angiogenesis (such as tumor growth, tumor invasion or tumor metastasis) comprising administering to the subject an effective amount of the above pharmaceutical composition.

Another embodiment is a diagnostically useful composition for targeting angiogenic endothelial cells, comprising (a) the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative which is diagnostically labeled; and (b) a diagnostically acceptable carrier. Detectable labels include a radionuclide, a PET-imageable agent, a fluorescer, a fluorogen, a chromophore, a chromogen, a phosphorescer, a chemiluminescer or a bioluminescer. Preferred diagnostic radionuclides are ^{3}H, ^{14}C, ^{35}S, ^{99}Tc, ^{123}I, ^{125}I, ^{131}I, ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr and ^{201}Tl. Useful fluorescers or fluorogens are is fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, fluorescamine, a fluorescein derivative, Oregon Green, Rhodamine Green, Rhodol Green or Texas Red.

The above diagnostic composition is used in a method for detecting the presence of angiogenic endothelial cells (i) in a tissue, (ii) in an organ or (iii) in a biological sample, which tissue, organ or sample is suspected of having angiogenically-activated endothelial cells. The method comprises (a) contacting the tissue, organ or sample with the diagnostic composition; and (b) detecting the presence of the label associated with the tissue, organ or sample. Preferably, the contacting is *in vivo*. Both the contacting and the detecting may be *in vivo*. The invention is also directed to an affinity ligand useful for binding to angiogenic endothelial cells or to a D5 domain binding site. These ligands comprise the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative immobilized to a solid support or carrier. The ligand is used in a method for isolating a D5 domain binding molecule from a complex mixture, the method comprising: (a) contacting the mixture with the affinity ligand; (b) allowing any of the binding molecules to bind to the ligand; (c) removing unbound material from the ligand; and (d) eluting the bound D5 domain binding material.

Also provided is a method for isolating or enriching cells expressing D5 domain binding sites from a cell mixture, comprising (a) contacting the cell mixture with the affinity ligand or the above polypeptide, fusion polypeptide, fragment, homologue or functional derivative; (b) allowing any binding site-expressing cell to bind to the ligand or the polypeptide, fusion polypeptide, fragment, homologue or functional derivative; (c) separating cells bound to the

ligand, polypeptide, fusion polypeptide, fragment, homologue or functional derivative from unbound cells; and (d) removing the bound cells, thereby isolating or enriching the D5 domain binding site-expressing cells.

A method for isolating or enriching cells expressing D5 domain binding sites from a cell mixture comprises (a) contacting the cell mixture with the affinity ligand of claim 57; (b) allowing any D5- -expressing cell to bind to the ligand; (c) removing unbound cells from the ligand and from the bound cells; and (d) releasing the bound cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Primary sequence and genetic structure of high molecular weight kininogen. Numbers 1-626 are amino acid (aa) locations with leader sequence -18 to -1. Letters A-J are the locations of intron/exon junctions. Domain 1 (aa 1-113) is coded by exons 1, 2 and 3. Domain 2 (aa 114-234) is coded by exons 4, 5 and 6. Domain 3 (aa 235-357) is coded by exons 7, 8 and 9. Domain 4 (aa 358-383) is coded by exon 10_{BK}. Domain 5 (aa 384-502) is coded by the 5' portion of exon 10_{HK}. Domain 6 (aa-503-626) is coded by the 3' portion of exon 10_{HK}. Curved arrows indicate kallikrein cleavage sites. Boxed "O" and "N" are the locations of O- and N-linked carbohydrate chains, respectively. XI is the putative factor XI binding sequence. PK is the putative prekallikrein binding sequence (from DeLa Cadena [47]).

Figure 2A, 2B and 2C is a set of gels and blots showing the identification and purification of CBP-D5 expressed in *E. coli*. *E. coli* were transformed with an expression vector containing the cDNA for CBP-D5. Small scale cultures were grown at 30°C and induced with IPTG. Cells were extracted and the supernatants and inclusion bodies analyzed by western blot (Fig. 2A) using an antibody previously raised against D5and SDS-PAGE (Fig 2B). The CBP-D5 constituted most of the protein in the inclusion bodies as demonstrated by SDS-PAGE (Fig. 2B). Inclusion bodies were extracted and the protein was re-folded and purified as described in the Examples. Fractions eluted from a calmodulin column (CAM) containing pure protein are shown in Fig. 2C.

Figure 3: Inhibition of EC proliferation by D5 and CBP-D5. HUVEC in EBM media (3,000 cells/well) were added to 96 well plates coated with gelatin. The cells were allowed to adhere for 4 hours at which time the EBM media was exchanged for EBM+bFGF (10 ng/mL) + inhibitor. The plates were allowed to incubate for 48 hours at which time the total cell number per each well was determined using the MTS assay (n=3 per each concentration of inhibitor).

Figure 4: Inhibition of angiogenesis in the CAM model using various D5 fusion constructs. Chick eggs were incubated for 7 days at which time the top of the egg was carefully removed to expose the chorioallantoic membrane (CAM). Disks impregnated with either bFGF alone (30 ng) or bFGF+HKa (ATN-234) or bFGF+HKa D5 (ATN-235) were placed on the CAM and the eggs incubated an additional 4 days. Neovessel formation (angiogenesis) was evaluated under a dissecting microscope by counting the number of vessels adjacent to the disk (n=3 for each condition).

Figure 5: Inhibition of EC tube formation on Matrigel®. Matrigel® (0.1 mg/mL) was plated into 96 well plates. bFGF (10 ng/mL), VEGF (1 ng/mL) and PMA (20 nM) were combined to stimulate tube formation in the presence of HUVEC (3000 cells/well). HKa D5 (ATN-235) was added in the presence of equimolar amounts of low molecular weight heparin (which stimulates the activity of ATN-235) at the outset of the assay. The plates were evaluated by two independent readers after 24 hours of incubation at 37°C.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

General References

Unless otherwise indicated, the practice of many aspects of the present invention employs conventional techniques of molecular biology, recombinant DNA technology and immunology, which are within the skill of the art. Such techniques are described in more detail in the scientific literature, for example, Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989, Ausubel, F.M. *et al.* *Current Protocols in Molecular Biology*, Wiley-Interscience, New York, current volume; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Lewin, BM, *Genes IV*, Oxford University Press, Oxford, (1990); Watson, J.D. *et al.*, *Recombinant DNA*, Second Edition, Scientific American Books, New York, 1992; Darnell, JE *et al.*, *Molecular Cell Biology*, Scientific American Books, Inc., New York, NY (1986); Old, R.W. *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press, Berkeley, CA (1981); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., Current Edition); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., Current Edition); *Transcription and Translation* (B. Hames & S. Higgins, eds., Current Edition); *Methods in Enzymology: Guide to Molecular Cloning Techniques*, (Berger and Kimmel, eds., 1987); Hartlow, E. *et al.*, *Antibodies: A*

Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988), Coligan, J.E. *et al.*, eds., *Current Protocols in Immunology*, Wiley-Interscience, New York 1991. Protein structure and function is discussed in Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, TE, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983.

PROTEINS, POLYPEPTIDES AND PEPTIDES

The full sequence of the mature form of HK (SEQ ID NO:1) is presented below.

```
QESQSEEIDC NDKDLFKAVD AALKKYNSQN QSNNQFVLYR ITEATKTVGS DTFYSFKYEI    60
KEGDCPVQSG KTWQDCEYKD AAKAATGECT ATVGKRSSTK FSVATQTCQI TPAEGPVVTA   120
QYDCLGCVHP ISTQSPDLEP ILRHGIQYFN NNTQHSSLFM LNEVKRAQRQ VVAGLNFRIT   180
YSIVQTNCSK ENFLFLTPDC KSLWNGDTGE CTDNAYIDIQ LRIASFSQNC DIYPGKDFVQ   240
PPTKICVGCP RDIPTNSPEL EETLTHTITK LNAENNATFY FKIDNVKKAR VQVVAGKKYF   300
IDFVARETTC SKESNEELTE SCETKKLGQS LDCNAEVYVV PWEKKIYPTV NCQPLGMISL   360
MKRPPGFSPF RSSRIGEIKE ETTVSPPHTS MAPAQDEERD SGKEQGHTRR HDWGHEKQRK   420
HNLGHGHKHE RDQGHGHQRG HGLGHGHEQQ HGLGHGHKFK LDDDLEHQGG HVLDHGHKHK   480
HGHGHGKHKN KGKKNGKHNG WKTEHLASSS EDSTTPSAQT QEKTEGPTPI PSLAKPGVTV   540
TFSDFQDSDL IATMMPPISP APIQSDDDWI PDIQTDPNGL SFNPISDFPD TTSPKCPGRP   600
WKSVSEINPT TQMKESYYFD LTDGLS                                        626
```

The present inventors have discovered that a polypeptide corresponding to the D5 domain of HK (amino acid residues 384-508 of the mature HK sequence; underscored above) is useful as an inhibitor of angiogenesis and of various EC functions including cell proliferation.

The 125 residue D5 domain therefore has the sequence SEQ ID NO:2:

```
VSPPHTSMAP AQDEERDSGK EQGHTRRHDW GHEKQRKHNL GHGHKHERDQ    50
GHGHQRGHGL GHGHEQQHGL GHGHKFKLDD DLEHQGGHVL DHGHKHKHGH   100
GHGKHKNKGK KNGKHNGWKT EHLAS                               125
```

Takagaki,Y *et al.*, *J. Biol. Chem. 260:*8601-8609 (1985) disclosed a cDNA clone encoding mature form of HK. The D5 coding region of this molecule is SEQ ID NO:3) is:

```
gta agt cca ccc cac act tcc atg gca cct gca caa gat gaa gag cgg
gat tca gga aaa gaa caa ggg cat act cgt aga cat gac tgg ggc cat
gaa aaa caa aga aaa cat aat ctt ggc cat ggc cat aaa cat gaa cgt
gac caa ggg cat ggg cac caa aga gga cat ggc ctt ggc cat gga cac
gaa caa cag cat ggt ctt ggt cat gga cat aag ttc aaa ctt gat gat
gat ctt gaa cac caa ggg ggc cat gtc ctt gac cat gga cat aag cat
aag cat ggt cat ggc cac gga aaa cat aaa aat aaa ggc aaa aag aat
gga aag cac aat ggt tgg aaa aca gag cat ttg gca agc
```

In addition to the native D5 domain polypeptide itself, fragments, variants, fusion polypeptides, or other functional derivatives of D5 including chemical derivatives and

peptidomimetics are used for the same purpose. For the sake of brevity, this entire group of compounds is collectively termed "**D5 polypeptides**" herein.

The D5 polypeptides bind to ECs at a binding site or receptor, which is operationally termed a "D5 binding site" or "D5BS." Binding of a D5 polypeptide to a D5BS on an EC:

(1) activates one or more signaling pathways leading to induction of EC apoptosis or

(2) inhibits a signaling pathway required for maintenance of EC viability.

The activation or inhibition of these pathways may be direct (as a result of binding to a D5BS) or indirect (result due to displacement of an EC from the matrix architecture). In either case, the net effect is loss of EC viability.

The peptides disclosed in Zhang *et al., supra,* also provide scaffolds for peptidomimetic design and structure-based drug design as well as the development of orally-active anti-angiogenic molecules which may provide a novel approach to achieving therapeutic anti-angiogenic effects. Thus, one objective of the present invention is development of HK-derived peptides as peptide-based drugs or peptidomimetic-based drugs.

Functional Derivatives of D5

A "functional derivative" retains measurable D5 activity, preferably that of binding to a D5BS on an EC and activating a biochemical process leading to EC apoptosis or inhibiting a process required for maintenance of EC viability, which permits its utility in accordance with the present invention. "Functional derivatives" encompass "mutants," "variants," "fragments," "analogues" or "chemical derivative" of D5, defined herein, regardless of whether the terms are used in the conjunctive or the alternative herein.

A "fragment" of D5 refers to any subset of the molecule, that is, a shorter peptide.

A "variant" of D5 refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of D5 refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein. A number of processes (chemical and recombinant) wall-known in the art can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the D5 protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

The term "mutant" is used interchangeably with "variant."

An "analogue" of D5 refers to a non- natural molecule substantially similar to either the entire molecule or a fragment thereof.

A "chemical derivative" of D5 contains additional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

A preferred functional derivative is a fusion polypeptide, a polypeptide that includes a D5 or functional fragment thereof. These are described in a separate section below.

A functional "homologue" of D5 must possess the biochemical and biological activities described above for D5. In view of this functional characterization, use of homologous proteins to D5 from other species, including proteins not yet discovered, fall within the scope of the invention if these proteins have the sequence similarities and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence, here, the human D5 protein (SEQ ID NO:2). The amino acid residues (or nucleotides) at corresponding amino acid positions (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the

percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol. 48*:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to human or murine *D5 nucleic acid molecules*. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to human or murine D5 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*,, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of the D5 protein described above is characterized as having (a) functional activity of the reference D5, and (b) sequence similarity to a "native" D5 protein (such as SEQ ID NO:2), when determined above, of at least about 30% (at the amino acid level), preferably at least about 50%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of D5. Then, the protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein. A biological

assay will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A preferred group of D5 variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions which may be made in a D5 polypeptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following groups:

Also included in this invention are D5 peptide variants in which at least one amino acid residue and preferably, only one, has been removed and a different residue inserted in its place. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1979, and Creighton, T.E., *Proteins: Structure and Molecular Principles*, W.H. Freeman & Co., San Francisco, 1984, which are hereby incorporated by reference. The types of substitutions which may be made in the peptide molecule of the present invention are conservative substitutions and are defined herein as exchanges within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: *e.g.*, Ala, Ser, Thr, Gly;
2. Polar, negatively charged residues and their amides: *e.g.*, Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: *e.g.*, His, Arg, Lys;

Pro, because of its unusual geometry, tightly constrains the chain. Substantial changes in functional properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above groups (or two other amino acid groups not shown above), which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Most substitutions according to the present invention are those which do not produce radical changes in the characteristics of the peptide molecule. Even when it is difficult to predict the exact effect of a substitution in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine

screening assays, preferably the biological and biochemical assays described herein. Modifications of peptide properties including redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

Preferred variants are those that have increased stability. These changes are based on NMR and molecular dynamic analysis, where one examines regions that are defined by the active site of D5. The capacity for motion in these structures is considered along with the impact of restraining the motion at particular sites on rigidity and biological activity of the molecule. Conventional approaches of protein engineering are applied. In one embodiment, stability is increased by introducing one or more Cys residues into strategic positions , where the formation of disulfide bonds between two Cys residues increases stability. Another approach is based on introduction of residues that form α helices at sites that do not impede the polypeptide's biological activity, for example at the N- and C- termini. These helices have a charged face and a hydrophobic face, and because of the highly charged nature of the polypeptide, hydrophobic residues in the helices will enter into from helix-helix interactions that further stabilize the polypeptide.

Chemical Derivatives of D5

"Chemical derivatives" of D5 contain additional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Capped polypeptides discussed below are examples of preferred chemical derivatives of a "natural" uncapped polypeptide. Any of the present combination of substitution or addition variants may be capped with any of the capping groups disclosed herein.

Other examples of chemical derivatives of the polypeptide follow.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal;

chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino group of lysine (Creighton, *supra,* pp. 79-86), acetylation of the N-terminal amine, and amidation of the C-terminal carboxyl groups.

Multimeric Peptides

The present invention also includes longer peptides in which a sequence from D5 or a variant thereof is repeated from about two to about 100 times, with or without intervening spacers or linkers. A multimer of the peptide referred to symbolically in this section as ***D5*** is shown by the following formula

$(\underline{D5}\text{-}X_m)_n\text{-}\underline{D5}$ wherein m= 0 or 1, n = 1-100. X is a spacer group, consisting of 1-20 glycine residues or chemical cross-linking agents

It is understood that such multimers may be built from any of the peptide variants described herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers and the disclosed substitution variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced chemically, the oligomers preferably have from 2-8 repeats of the basic polypeptide sequence. When produced recombinantly, the multimers may have as many repeats as the expression system permits, for example from two to about 100 repeats.

The multimer of a D5 sequence can further be fused to another polypeptide, resulting in fusion polypeptides that include more than a single repeat of the D5 sequence..

Capped Polypeptides

Any D5 polypeptide may be blocked or capped at its amino and carboxyl termini, preferably with acetyl bound to the amino-terminal N ("Ac") and amido (-NH_2 bound to the C-terminal carboxyl group ("Am")), respectively. The N-terminal capping function is preferably linked to the terminal amino function and may be selected from the group consisting of:

formyl; alkanoyl, having from 1 to 10 carbon atoms, such as acetyl, propionyl, butyryl; alkenoyl, having from 1 to 10 carbon atoms, such as hex-3-enoyl; alkynoyl, having from 1 to 10 carbon atoms, such as hex-5-ynoyl; aroyl, such as benzoyl or 1-naphthoyl; heteroaroyl, such as 3-pyrroyl or 4-quinoloyl; alkylsulfonyl, such as methanesulfonyl; arylsulfonyl, such as benzenesulfonyl or sulfanilyl; heteroarylsulfonyl, such as pyridine-4-sulfonyl;

substituted alkanoyl, having from 1 to 10 carbon atoms, such as 4-aminobutyryl;

substituted alkenoyl, having from 1 to 10 carbon atoms, such as 6-hydroxy-hex-3-enoyl;

substituted alkynoyl, having from 1 to 10 carbon atoms, such as 3-hydroxy-hex-5-ynoyl;

substituted aroyl, such as 4-chlorobenzoyl or 8-hydroxy-naphth-2-oyl;

substituted heteroaroyl, such as 2,4-dioxo-1,2,3,4-tetrahydro-3-methyl-quinazolin-6-oyl;

substituted alkylsulfonyl, such as 2-aminoethanesulfonyl;

substituted arylsulfonyl, such as 5-dimethylamino-1-naphthalenesulfonyl;

substituted heteroarylsulfonyl, such as 1-methoxy-6-isoquinolinesulfonyl; carbamoyl or thiocarbamoyl;

substituted carbamoyl (R'-NH-CO) or substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkyl, alkenyl, alkynyl, aryl, heteroaryl, substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, or substituted heteroaryl;

substituted carbamoyl (R'-NH-CO) or substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkanoyl, alkenoyl, alkynoyl, aroyl, heteroaroyl, substituted alkanoyl, substituted alkenoyl, substituted alkynoyl, substituted aroyl, or substituted heteroaroyl, all as above defined;

The C-terminal capping function can either be in an amide bond with the terminal carboxyl or in an ester bond with the terminal carboxyl. Capping functions that provide for an amide bond are designated as NR^1R^2 wherein R^1 and R^2 may be independently drawn from the following group:

hydrogen;

alkyl, preferably having from 1 to 10 carbon atoms, such as methyl, ethyl, isopropyl;

alkenyl, preferably having from 1 to 10 carbon atoms, such as prop-2-enyl;

alkynyl, preferably having from 1 to 10 carbon atoms, such as prop-2-ynyl;

substituted alkyl having from 1 to 10 carbon atoms, such as hydroxyalkyl, alkoxyalkyl, mercaptoalkyl, alkylthioalkyl, halogenoalkyl, cyanoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkanoylalkyl, carboxyalkyl, carbamoylalkyl;

substituted alkenyl having from 1 to 10 carbon atoms, such as hydroxyalkenyl, alkoxyalkenyl, mercaptoalkenyl, alkylthioalkenyl, halogenoalkenyl, cyanoalkenyl, aminoalkenyl, alkylaminoalkenyl, dialkylaminoalkenyl, alkanoylalkenyl, carboxyalkenyl, carbamoylalkenyl;

substituted alkynyl having from 1 to 10 carbon atoms, such as hydroxyalkynyl, alkoxyalkynyl, mercaptoalkynyl, alkylthioalkynyl, halogenoalkynyl, cyanoalkynyl, aminoalkynyl, alkylaminoalkynyl, dialkylaminoalkynyl, alkanoylalkynyl, carboxyalkynyl, carbamoylalkynyl;

aroylalkyl having up to 10 carbon atoms, such as phenacyl or 2-benzoylethyl;

aryl, such as phenyl or 1-naphthyl;

heteroaryl, such as 4-quinolyl;

alkanoyl having from 1 to 10 carbon atoms, such as acetyl or butyryl;

aroyl, such as benzoyl;

heteroaroyl, such as 3-quinoloyl;

OR' or NR'R'' where R' and R'' are independently hydrogen, alkyl, aryl, heteroaryl, acyl, aroyl, sulfonyl, sulfinyl, or SO_2-R''' or SO-R''' where R''' is substituted or unsubstituted alkyl, aryl, heteroaryl, alkenyl, or alkynyl.

Peptidomimetics

Another class of compounds useful in this regard are low molecular weight peptidomimetic compounds which influence the interactions between a D5 polypeptide and the D5BS. Such peptidomimetics may - be derived from the structure of either the free D5 or D5 bound to D5BS.

A peptidomimetic of D5 mimics the biological effect of a D5 polypeptide and may be an unnatural peptide or a non-peptide agent which has the stereochemical properties of a D5 polypeptide such that it has the binding activity or biological activity of the peptide. Hence, this invention includes compounds wherein a peptidomimetic compound is coupled to another peptide.

A peptidomimetic agent may be an unnatural peptide or a non-peptide agent which recreates the stereospatial properties of the binding elements of D5 such that it has the binding activity or biological activity of D5. Similar to the linear peptides corresponding to D5, a peptidomimetic will have a binding face (which interacts with the D5BS) and a non-binding face. Again, similar to linear peptides of D5, the non-binding face of a peptidomimetic will

contain functional groups which can be modified by various therapeutic moieties without modifying the binding face of the peptidomimetic. A preferred embodiment of a peptidomimetic would contain an aniline on the non-binding face of the molecule. The NH_2-group of an aniline has a pKa ~ 4.5 and could therefore be modified by any NH_2-selective reagent without modifying any NH_2 functional groups on the binding face of the peptidomimetic. Other peptidomimetics may not have any NH_2 functional groups on their binding face and therefore, any NH_2, without regard for pK_a could be displayed on the non-binding face as a site for conjugation. In addition other modifiable functional groups, such as -SH and -COOH could be incorporated into the non-binding face of a peptidomimetic as a site of conjugation. A therapeutic moiety could also be directly incorporated during the synthesis of a peptidomimetic and preferentially be displayed on the non-binding face of the molecule.

This invention also includes compounds, which retain partial peptide characteristics. For example, any proteolytically unstable bond within a peptide of the invention could be selectively replaced by a non-peptidic element such as an isostere (N-methylation; D-amino acid at a particular site) or a reduced peptide bond while the rest of the molecule retains its peptide nature.

Peptidomimetic compounds, either agonists, substrates or inhibitors, have been described for a number of bioactive peptides such as opioid peptides, VIP, thrombin, HIV protease, *etc.* Methods for designing and preparing peptidomimetic compounds are known in the art (Hruby, V.J., *Biopolymers 33*:1073-1082 (1993); Wiley, R.A. *et al.*, *Med. Res. Rev. 13*:327-384 (1993); Moore *et al.*, *Adv. in Pharmacol 33*:91-141 (1995); Giannis *et al.*, *Adv. in Drug Res. 29*:1-78 (1997), which references are incorporated by reference in their entirety). These methods are used to make peptidomimetics that possess at least the binding capacity and specificity of the cyclic peptides and preferably also possess the biological activity. Knowledge of peptide chemistry and general organic chemistry available to those skilled in the art are sufficient, in view of the present disclosure, for designing and synthesizing such compounds.

For example, such peptidomimetics may be identified by inspection of the cystallographically-derived three-dimensional structure of a peptide of the invention either free or bound in complex with a D5BS. Alternatively, the structure of a peptide of the invention bound to D5BS can be gained by using nuclear magnetic resonance spectroscopy. The better knowledge of the stereochemistry of the interaction of a peptide with its binding partner will permit the rational design of such peptidomimetic agents.

D5 Fusion Polypeptides that Inhibit Angiogenesis

Fusion polypeptides of D5 have been prepared and tested for anti-angiogenic activity. Fusions with thioredoxin, calmodulin binding protein (CBP), maltose-binding protein (MBP) and glutathione-S-transferase (GST) were prepared and expressed in *E. coli*.

A small amount of each polypeptide is expressed in soluble, active form that can be purified from the *E. coli* inclusion body fraction.

The present inventors developed a high-yield method for extracting and refolding the fusion polypeptides from inclusion bodies, thus making this method useful for preparing a D5 fusion polypeptide for therapeutic use.

One liter of *E. coli* culture grown to a density of 0.6 OD units/mL is in the range of 50-100 mg of the active refolded polypeptide. The bacteria is precipitated into a pellet by centrifugation and the pellet lysed using lysozyme followed by sonication to disrupt the cell membranes. Inclusion bodies are purified from the lysate and extracted in 6 M G

Guanidine HCl in 20 mM Tris HCl buffer pH 7.5. The extracted polypeptide is allowed to refold by slow dilution (0.1 mL/minute into 300 mL) into 20 mM Tris HCl pH 7.5 followed by stirring at 4 C for 24 hrs. Refolded polypeptide remains in solution under these conditions and misfolded and denatured polypeptide precipitates and can be removed by filtration or centrifugation. The further purification of D5 depends on the identity of the fusion partner and can be achieved using affinity or ion exchange chromatography. For example, the CBP-D5 fusion is predicted to have a pI of 9.0 and can thus be purified using cation exchange such as SP (sulfopropyl)-Sepharose developed at pH 8.5. Very few proteins have as basic of a pI as CBP-D5 and thus, only CBP-D5 would be positively charged at pH and capable of sticking to the column. This protocol has been used for the one-step purification of the CBP-D5 fusion to homogeneity.

An active D5 fusion polypeptide can be expressed and purified in this manner regardless of fusion partner. Fusion polypeptides, as well as pure D5 cleaved from the fusion polypeptide, inhibit the proliferation of ECs (IC_{50}= 60nM) and lead in these cells to rapid induction of apoptosis (about 4 hrs to DNA laddering).

The D5 fusion polypeptides are angiogenic EC-selective in that they have no measurable effect on the proliferation of other cells such as aortic smooth muscle cells and liver cells (HepG2). The D5 fusion polypeptides do not affect the proliferation of non-stimulated (quiescent) ECs.

D5 also inhibits blood vessel formation in the CAM assay.

On the basis of these activities, a D5 fusion polypeptide that binds to the D5BS on ECs and has any of the above inhibitory actions on ECs will prevent the angiogenesis that is requisite for tumor growth and will therefore exert an antitumor activity in a subject

In a preferred D5 fusion partner, the binding partner is fused to the N-terminus of D5.

A preferred D5 fusion polypeptide has a cleavable linker between the D5 portion and the fusion partner. Examples of cleavable linkers include VPRGSD (SEQ ID NO:9) and DDKDWH (SEQ ID NO:10, cleavable by thrombin; DDKDWH, cleavable by enterokinase. Any sequence with a basic residue (K or R) in the P1 position is potentially cleavable by thrombin, enterokinase, trypsin or plasmin. However, thrombin is the least promiscuous of these enzymes and is preferred.

In one embodiment, the fusion polypeptide comprises a sequence from D5 and more than one additional protein. Thus, in a fusion polypeptide as described above, the fusion partner serves as a bridge to yet another sequence that serves as a diagnostic label or as a therapeutic moiety. By distancing such a label or moiety from the D5 structure, the probability increases that the ability of the D5 portion of the fusion polypeptide to bind to a D5BS will remain intact. Diagnostic labels and therapeutic moieties are disclosed in more detail below.

All the foregoing polypeptides, as well as their variants and chemical derivatives, including peptidomimetics, must bind to endothelial cells or a fraction of these cells that contains the D5BS, preferably with an $IC_{50} \leq 10\mu M$, more preferably $\leq 1\mu M$. This activity is characterized in greater detail below.

All the foregoing polypeptides, fusion polypeptides or other functional derivatives and chemical derivatives including peptidomimetics and multimeric peptides must have the biological activity or biochemical (*e.g.*,, binding activity) of the native D5 domain polypeptide of HK as follows: at least about 20% of the activity of native D5 in an *in vitro* assay of endothelial cell growth or cell viability or of angiogenesis. Alternatively, or in addition, these derivatives should compete with labeled D5 polypeptide (with an $IC_{50} \leq 10\mu M$, more preferably $\leq 1\mu M$) for binding to a ligand or binding partner, preferably the D5BS, when tested in a binding assay with whole endothelial cells or cell fractions, an isolated D5BS-containing polypeptide or peptide, or any other such binding molecule.

RECOMBINANT EXPRESSION OF D5 AND FUSION POLYPEPTIDES THEREOF

D5 polypeptides and fusion polypeptides are preferably produced using conventional recombinant DNA techniques

Expression Vectors and Host Cells

This invention includes an expression vector comprising a nucleic acid sequence encoding a D5 polypeptide operably linked to at least one regulatory sequence. "Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology*, vol. 185, Academic Press, San Diego, Calif. (1990)).

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of D5: full length protein and its functional derivatives (defined herein) including polypeptide fragments, variants, fusion polypeptides, *etc.* Thus, in one embodiment, the expression vector comprises a nucleic acid encoding at least a portion of the D5 protein, alone or fused to another protein.

Such expression vectors are used to transfect host cells for expression of the DNA and production of the encoded polypeptides which include fusion polypeptides or peptides. It will be understood that a genetically modified cell expressing the D5 polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose. The length of time that expression is required, or that the cell remain alive, is the time necessary for the cell to exert its stimulatory or inhibitory function. For example, in the case of a transduced cell expressing D5, expression of D5 may be for as little as 6 hours, preferably 24 hours, more preferably for at least 2-4 days. Of course, expression may also be stable (*i.e.*, for the life of the cell). Appropriate expression vectors and regulatory elements (*e.g.*, inducible or constitutive promoters) discussed below are selected in accordance with the desired or required stability of expression.

The present in invention provides methods for producing the D5 polypeptide, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes at least a portion of the D5 protein is cultured under appropriate conditions to allow expression of D5 polypeptide.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the D5 protein and DNA encoding at least a portion of a second protein, so that the host cells produce fusion polypeptides that include both the portions.

When the recombinant expression vector comprises DNA encoding a portion of D5 and DNA encoding another protein, the resulting fusion polypeptide may have altered solubility, binding affinity and/or valency. A D5 Ig fusion polypeptide, for example, is preferably secreted by transfected host cells in cultures and is therefor isolated from the culture medium. Alternatively, if protein is retained in the cytoplasm, the cells are harvested and lysed and the protein isolated from this lysate.

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. D5 protein can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology,* 22: 233-577 (1971)). Once purified, partially or to homogeneity, the recombinant D5 polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

Prokaryotic or eukaryotic host cells transformed or transfected to express D5 or a homologue or functional derivative thereof are within the scope of the invention. For example, D5 may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan *et al.* (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.).

Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3: 2156-2165,) and the pVL series (Lucklow, V. A., and Summers, M. D., (1989) *Virology* 170: 31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23: 175-182) are used in conjunction with such vectors as pCDM 8 (Aruffo A. and Seed, B., *supra,* for transient amplification/expression in mammalian cells, while CHO (*dhfr*-negative CHO) cells are used with vectors such as pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6: 187-195) for stable amplification/expression in mammalian cells. The NS0 myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the two fusion partner polypeptides to enable separation of the target protein from the partner sequence after purification of the fusion polypeptide. As discussed herein, proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69: 301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

One embodiment of this invention is a transfected cell which expresses D5 *de novo*. In the case of a cell already expressing D5, the transfected cell expresses increased amounts of D5 proteins or fragments thereof. For example, a tumor cell such as a sarcoma, melanoma, leukemia, lymphoma, carcinoma or neuroblastoma is transfected with an expression vector directing the expression of D5 on the tumor cell surface.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, e.g., 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the dNTPs. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tet Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using an excess of polynucleotide kinase to 1 nmole substrate in the presence of appropriate buffers, salts, *etc.*, and γ-^{32}P-ATP.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. In general, about 1 mg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 ml of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times

of about one hour to two hours at about 37°C. are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four dNTPs using known concentrations, incubation times and conditions. The Klenow fragment fills in at 5′ single-stranded overhangs but chews back protruding 3′ single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Ligations are typically performed in 15-50 ml volumes under conditions and temperatures known to be appropriate for "sticky end" or "blunt end" ligation.

In vector construction employing "vector fragments", the fragment is commonly treated with bacterial alkaline phosphatase or calf intestinal alkaline phosphatase to remove the 5' phosphate and prevent self-ligation. Re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of D5 DNA sequence (cDNA or genomic DNA) are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al., Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al., DNA* (1983) 2:183-193)). Correct ligations for plasmid construction are confirmed, for example, by first transforming *E. coli* strain MC1061 (Casadaban, M., *et al., J Mol Biol* (1980) 138:179-207) or other suitable host with the ligation mixture. Using conventional methods, transformants are selected based on the presence of the ampicillin-, tetracycline- or other

antibiotic resistance gene (or other selectable marker) depending on the mode of plasmid construction. Plasmids are then prepared from the transformants with optional chloramphenicol amplification optionally following chloramphenicol amplification ((Clewell, DB *et al.* , *Proc Natl Acad Sci USA* (1969) 62:1159; Clewell, D. B., *J Bacteriol* (1972) 110:667). Several mini DNA preps are commonly used. See, *e.g.*,, Holmes, DS, *et al.*, *Anal Biochem* (1981) 114:193-197; Birnboim, HC *et al.* , *Nucleic Acids Res* (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al.*, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al. Methods in Enzymology* (1980) 65:499.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69: 301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter

sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Suitable promoters may be inducible, repressible or constitutive. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No.

5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency on the D5-encoding DNA molecule of the present invention.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42C. in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

The present invention also includes cells containing and/or expressing the DNA encoding the D5 polypeptides of the present invention, including prokaryotic and eukaryotic cells and in particular, bacterial, plant, yeast, worm, insect, mouse or other rodent, and other mammalian cells, including human cells, of various types and lineages, whether frozen or in active growth, whether in culture or in a whole organism containing them.

Preferred mammalian cells may be primate, particularly human, but can be associated with any animal of interest, particularly domesticated animals, such as equine, bovine, murine,

ovine, canine, feline, *etc.* Among these species, various types of cells can be used such as hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle, spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, *etc.* Hematopoietic cells may be lymphoid or myelomonocytic lineages, including T and B lymphocytes. macrophages and monocytes, myoblasts and fibroblasts. Also of interest are stem and progenitor cells.

DELIVERY OF D5 DNA TO CELLS AND ANIMALS

DNA delivery, for example to effect what is also known colloquially as "gene therapy" involves introduction of a "foreign" DNA into a cell and ultimately, into a live animal. Several general strategies for gene therapy have been studied and have been reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460 (1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest. 7*:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the D5 expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an indefinite period of time (Wolff, J.A. *et al.*, *Science 247*:1465 (1990); Acsadi, G. *et al.*, *The New*

Biologist 3:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem.* *265*:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy* 2:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science* 252:431 (1991); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease,* Vol 1, Boehringer Manneheim Biochemicals, USA, 1991).

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy* 1:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills, J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol. 10*:4239 (1990). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, *i.e.*, actively growing tumor cells. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

The DNA molecules encoding the D5 sequences may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, R.F. *et al.*, *Cell* 33:153-159 (1983); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985),; Sorge, J., *et al.*, *Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, R.A. *et al.*, *Nature* 320:257 (1986); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al.*, U.S. 5,278,056.

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel,

EG *et al.*, *Science 244*:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, M.S., In: *Virology*, Fields, BN *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, K.L., *Biotechniques 6*:616 9191988), Strauss, S.E., In: *The Adenoviruses*, Ginsberg, HS, ed., Plenum Press, New York, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene therapy include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, R.J. *et al.*, *EMBO J. 10*:3941 (1991) according to the present invention.

Another vector which can express the DNA molecule of the present invention, and is useful in the present therapeutic setting, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) *89*:10847-10851; Fuerst, T.R. *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) *86*:2549-2553; Falkner F.G. *et al.*; *Nucl. Acids Res* (1987) *15*:7192; Chakrabarti, S *et al.*, *Molec. Cell. Biol.* (1985) *5*:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) *3*:86-90; Moss, B. *Biotechnology* (1992) *20*: 345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) *158*:25-38; Moss, B., *Science* (1991) *252*:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* **291**, 238-239 (1981); Poirier, TP *et al. J. Exp. Med.* **168**, 25-32 (1988); (Sadoff, J.C., *et al., Science* **240**, 336-338 (1988); Stover, C.K., *et al., Nature* **351**, 456-460 (1991); Aldovini, A. *et al.,, Nature* **351**, 479-482 (1991); Schafer, R., *et al., J. Immunol.* **149**, 53-59 (1992); Ikonomidis, G. *et al., J. Exp. Med.* **180**, 2209-2218 (1994)).

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*,

1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA 87*:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 280*:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 244*:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol. 27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta 1088*:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al.*, *J. Biol. Chem. 264*:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem. 263*:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem. 267*:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA according to the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods.

Again, as noted above, for the utility of transduced D5 molecules according to this invention may not require stable expression. Rather, transient expression of the polypeptide may be sufficient for transduced cells to perform their biological/pharmacological function.

ASSAY METHODS

A number of conventional techniques useful for evaluating the biological and pharmacological activities of the D5 polypeptides are described below.

Endothelial Cell Proliferation.

HUVEC or HDMVEC (purchased from Cell Systems or Clonetics) are plated at a density of 3,000 cells per well in gelatin-coated 96 well plates. The cells are allowed to adhere and spread (4-6 hours at 37° C). The medium is then removed and replaced with fresh M199

containing 2% FBS, 10 ng/ml recombinant human bFGF and various concentrations of test peptide.

In selected studies, similar concentrations of other EC growth factors, such as VEGF, is used.

Cells are then be cultured for an additional 48 hours at 37° C, at which time the relative cell numbers in each well is determined using the Cell Titer® Aq_{ueous} cell proliferation assay (Promega). Briefly, 20 µl of a 19:1 (V/V) mixture of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) are be added to each well, and after an additional hour of incubation, A_{490} is measured.

The present inventor has quantitated ECs following incubation with HK_a using both this method and manual cell counting, and observed a direct linear correlation.

The percent inhibition of proliferation will be calculated as previously described (Zhang *et al.*, *supra*), using the formula:

$$\% \text{ inhibition} = \frac{(1 - (A_{490\,(+GF,\,HKa)} - A_{490\,(-GF)})}{(A_{490\,(+GF)} - A_{490\,(-GF)})} \times 100.$$

Endothelial Cell Migration

These studies are performed using a 96-well migration apparatus (Neuro-Probe). Briefly, wells of a 96-well microplate are filled with M199 containing 10 ng/ml VEGF. The microplate is then placed into an acrylic chamber, and a polycarbonate filter (0.8 µM pore size), precoated with fibronectin or type I collagen, is placed over the top of the microplate (this filter covers the entire plate). The hinged lid of the migration chamber, which contains 96 holes that align directly with the microplate wells, is then closed over the filter and clamped tightly. HDMVEC, which are significantly more active in migration assays than HUVEC, are added to the "wells" of the lid, at a concentration of 50,000 cells/well. The migration apparatus is then incubated at 37°C for 6 hours, at which time the filter is removed and stained with Giemsa. After washing, the filter is placed directly in a microplate reader, and the OD_{540} measured.

The sensitivity of this assay may be increased, and the time required for migration reduced, by preloading cells with Calcein-acetoxymethyl ester (Molecular Probes). This lipophilic compound traverses cell membranes, where intracellular hydrolysis releases the fluorescent, charged calcein moiety. Since the latter leaks from cells only slowly, it may be used to quantitate cells using a fluorescent plate reader. Percent inhibition is calculated as:

$$\frac{\text{(\# Migrated cells with VEGF + test agent}}{\text{(\#Migrated cells with VEGF only)}} \times 100$$

Endothelial cell apoptosis.

These studies will performed after identifying peptides which inhibit EC proliferation, to determine if, as with the parent molecule, these effects reflect the induction of EC apoptosis. Apoptosis is detected using DAPI staining, DNA fragmentation or Annexin 5 binding to treated cells. These methods will be performed as previously described (Zhang *et al.., supra*). Cells treated with D5 fusions or peptides derived from D5 rapidly commit to apoptosis (within 4-8 hours). HUVEC or HDMVEC (purchased from Cell Systems or Clonetics) are plated at a density of 3,000 cells per well in gelatin-coated 96 well plates. The cells are allowed to adhere and spread (4-6 hours at 37° C). The medium is then removed and replaced with fresh M199 containing 2% FBS, 10 ng/ml recombinant human bFGF and various concentrations of test peptide or D5 fusion. After 4-8 hours of treatment, the cells are harvested (for DNA fragmentation) or stained with DAPI (which fluoresces more brightly in cells with fragmented DNA). Alternatively, the amount of fluorescent Annexin 5 binding, which binds to the phosphatidylserine that is exposed to the extracellular face of the cell surface in apoptotic cells, is measured in a fluorescent plate reader. Quantitation is best achieved using the DAPI or Annexin 5 protocols and can be measured as an increase of fluorescence in the wells containing test agent compared to control. Agents known to induce endothelial cell apoptosis such as ursolic acid and TNP470 are included as a positive control. The DAPI increase in fluorescence can be correlated directly with the number of apoptotic cells by manual counting of cells with highly fluorescent nuclei.

Tube formation assay

ECs can be induced to form capillary-like tubes on Matrigel. Matrigel is dispensed into 96-well plates and allowed to gel. HUVECS or HMVECs (3-6,000 cells/well) are added to the plate in the presence of bFGF (10 ng/mL), VEGF (10 ng/mL) and PMA (75 nM) in addition to any test agent. The number of tubes formed per well are scored visually by at least 2 different readers. The values are expressed as percentage of control wells which receive no test agent.

***In Vivo* Evaluation of D5 Polypeptides**

Matrigel® Plug Assay

This assay is performed essentially as described by Passaniti *et al.* (*Lab Invest.* 67:519-528 (1992)). Matrigel® is maintained at 4°C until use. Just prior to injection, Matrigel® is mixed with angiogenic factors (100 ng/mL bFGF, 100 ng/mL VEGF), then injected s.c. into mice (0.5 mL per mouse). The injected Matrigel® forms a palpable solid gel which persists for 10 days, at which time the animals are euthanized. The Matrigel® plugs are removed and angiogenesis quantitated by measuring the amount of hemoglobin in the Matrigel® plugs or by counting neovessels in sections prepared from the plugs. Anti-CD31 staining may be used to confirm neovessel formation and microvessel density in the plugs. In some experiments, tumor cells may be mixed with the Matrigel in lieu of a specific angiogenic factor to investigate the ability of the test agents. In either configuration, the test agent is mixed directly into the Matrigel and can also be directly injected on a daily basis into the plug over the course of the experiment. In one orientation of the model, D5 or fusion D5 cDNA will be transfected into tumor cells in a secretable expression system. Expression and secretion of the protein will be confirmed in cell culture. D5 expressing tumor cells will then be mixed with Matrigel and evaluated for anti-angiogenic activity in the Matrigel Plug assay.

Chick Chorioallantoic Membrane (CAM) Angiogenesis Assay

This assay is performed essentially as described by Nguyen et al (*Microvascular Res.* 47:31-40 (1994)). A mesh containing either angiogenic factors (bFGF) or tumor cells plus inhibitors is placed onto the CAM of an 8-day old chick embryo and the CAM observed for 3-9 days after implantation of the sample. Angiogenesis is quantitated by determining the percentage of squares in the mesh which contain blood vessels.

In Vivo Testing of Compositions in Animal Models of Human Tumors

The peptides, peptidomimetics and conjugates are tested for therapeutic efficacy in several well established rodent models which are considered to be highly representative of a broad spectrum of human tumors. The approaches are described in detail in Geran, R.I. *et al.*, "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems (Third Edition)", *Canc. Chemother. Reports*, Part 3, 3:1-112, which is hereby incorporated by reference in its entirety. All general test evaluation procedures, measurements and calculations are performed in accordance with this reference, including mean survival time, median survival time, calculation of approximate tumor weight from measurement of tumor diameters with vernier calipers; calculation of tumor diameters;

calculation of mean tumor weight from individual excised tumors; and ratios between treated and control groups ratio for any measure (T/C ratios).

A. HT1080 xenograft model

HT1080 human fibrosarcoma tumor cells are passaged *in vitro*. Nude Balb/c mice are inoculated with 10^5- 10^6 tumor cells *sc* on the right flank. Primary tumor growth is evaluated using caliper measurements. The peptide or polypeptide being tested for antitumor efficacy may be dissolved in PBS and administered by continuous infusion or by daily iv, sc or ip injections. Control animals receive PBS alone. In another embodiment of this model, tumor cells are first transfected to a D5 polypeptide. These transfected cells inoculated into the animals and the growth of the transfected tumor cells is compared to non-transfected cells or cells transfected with an empty or control expression vector. This embodiment mimics local delivery of the test agent which would model the a gene delivery approach wherein cDNA encoding the D5 polypeptide was administered to the subject.

B. 3LL Lewis Lung Carcinoma: Primary Tumor Growth

This tumor line arose spontaneously in 1951 as carcinoma of the lung in a C57BL/6 mouse (*Cancer Res 15*:39, 1955. See, also Malave, I. *et al., J. Nat'l. Canc. Inst. 62*:83-88 (1979)). It is propogated by passage in C57BL/6 mice by subcutaneous (sc) inoculation and is tested in semiallogeneic C57BL/6 x DBA/2 F_1 mice or in allogeneic C3H mice. Typically six animals per group for subcutaneously (sc) implant, or ten for intramuscular (im) implant are used. Tumor may be implanted sc as a 2-4 mm fragment, or im or sc as an inoculum of suspended cells of about 0.5-2 x 10^6-cells. Treatment begins 24 hours after implant or is delayed until a tumor of specified size (usually approximately 400 mg) can be palpated. The test compound is administered ip daily for 11 days

Animals are followed by weighing, palpation, and measurement of tumor size. Typical tumor weight in untreated control recipients on day 12 after im inoculation is 500-2500 mg. Typical median survival time is 18-28 days. A positive control compound, for example cyclophosphamide at 20 mg/kg/injection per day on days 1-11 is used. Results computed include mean animal weight, tumor size, tumor weight, survival time For confirmed therapeutic activity, the test composition should be tested in two multi-dose assays.

C. 3LL Lewis Lung Carcinoma: Primary Growth and Metastasis Model

This model has been utilized by a number of investigators. See, for example, Gorelik, E. *et al., J. Nat'l. Canc. Inst. 65*:1257-1264 (1980); Gorelik, E. *et al., Rec. Results Canc. Res.*

75:20-28 (1980); Isakov, N. *et al.*, *Invasion Metas. 2*:12-32 (1982); Talmadge J.E. *et al.*, *J. Nat'l. Canc. Inst. 69*:975-980 (1982); Hilgard, P. *et al.*, *Br. J. Cancer 35*:78-86(1977)). Test mice are male C57BL/6 mice, 2-3 months old. Following sc, im, or intra-footpad implantation, this tumor produces metastases, preferentially in the lungs. With some lines of the tumor, the primary tumor exerts anti-metastatic effects and must first be excised before study of the metastatic phase (see also U.S. 5,639,725).

Single-cell suspensions are prepared from solid tumors by treating minced tumor tissue with a solution of 0.3% trypsin. Cells are washed 3 times with PBS (pH 7.4) and suspended in PBS. Viability of the 3LL cells prepared in this way is generally about 95-99% (by trypan blue dye exclusion). Viable tumor cells (*e.g.*, 3×10^4 - 5×10^6) suspended in 0.05 ml PBS are injected subcutaneously, either in the dorsal region or into one hind foot pad of C57BL/6 mice. Visible tumors appear after 3-4 days after dorsal sc injection of 10^6 cells. The day of tumor appearance and the diameters of established tumors are measured by caliper every two days.

The treatment is given as one or two doses of peptide or derivative, per week. In another embodiment, the peptide is delivered by osmotic minipump.

In experiments involving tumor excision of dorsal tumors, when tumors reach about 1500 mm^3 in size, mice are randomized into two groups: (1) primary tumor is completely excised; or (2) sham surgery is performed and the tumor is left intact. Although tumors from 500-3000 mm^3 inhibit growth of metastases, primary tumors of 1500 mm^3 are the largest that can be safely resected with high survival and without local regrowth. After 21 days, all mice are sacrificed and autopsied.

Lungs are removed, weighed, fixed in Bouin's solution and the number of visible metastases is recorded. The diameters of the metastases are also measured using a binocular stereoscope equipped with a micrometer-containing ocular under 8X magnification. On the basis of the recorded diameters, it is possible to calculate the volume of each metastasis. To determine the total volume of metastases per lung, the mean number of visible metastases is multiplied by the mean volume of metastases. To further determine metastatic growth, it is possible to measure incorporation of ^{125}IdUrd into lung cells (Thakur, M.L. *et al.*, *J. Lab. Clin. Med. 89*:217-228 (1977). Ten days following tumor amputation, 25 μg of fluorodeoxyuridine is inoculated into the peritoneums of tumor-bearing (and, if used, tumor-resected mice). After 30 min, mice are given 1 μCi of ^{125}IdUrd (iododeoxyuridine). One day later, lungs and spleens are removed and weighed, and a degree of ^{125}IdUrd incorporation is measured using a gamma counter.

In mice with footpad tumors, when tumors reach about 8-10 mm in diameter, mice are randomized into two groups: (1) legs with tumors are amputated after ligation above the knee joints; or (2) mice are left intact as nonamputated tumor-bearing controls. (Amputation of a tumor-free leg in a tumor-bearing mouse has no known effect on subsequent metastasis, ruling out possible effects of anesthesia, stress or surgery). Mice are killed 10-14 days after amputation. Metastases are evaluated as described above.

Statistics: Values representing the incidence of metastases and their growth in the lungs of tumor-bearing mice are not normally distributed. Therefore, non-parametric statistics such as the Mann-Whitney U-Test may be used for analysis.

Study of this model by Gorelik *et al.* (1980, *supra*) showed that the size of the tumor cell inoculum determined the extent of metastatic growth. The rate of metastasis in the lungs of operated mice was different from primary tumor-bearing mice. Thus in the lungs of mice in which the primary tumor had been induced by inoculation of larger doses of 3LL cells (1-5 x 10^6) followed by surgical removal, the number of metastases was lower than that in nonoperated tumor-bearing mice, though the volume of metastases was higher than in the nonoperated controls. Using ^{125}IdUrd incorporation as a measure of lung metastasis, no significant differences were found between the lungs of tumor-excised mice and tumor-bearing mice originally inoculated with 1 x 10^6 3LL cells. Amputation of tumors produced following inoculation of 1 x 10^5 tumor cells dramatically accelerated metastatic growth. These results were in accord with the survival of mice after excision of local tumors. The phenomenon of acceleration of metastatic growth following excision of local tumors had been repeatedly observed (for example, see U.S. 5,639,725). These observations have implications for the prognosis of patients who undergo cancer surgery.

For a compound to be useful in accordance with this invention, it should demonstrate biological or pharmacological activity in at least one of the *in vitro* or *in vivo* assay systems described herein.

Diagnostic and Prognostic Compositions

The D5 polypeptides of the invention have been designed so that they can be detectably labeled and used, for example, to detect a D5BS on the surface or in the interior of a cell. The fate of the D5 polypeptide during and after binding can be followed *in vitro* or *in vivo* by using the appropriate method to detect the label. The labeled D5 polypeptide may be utilized *in vivo* for diagnosis and prognosis

Because these polypeptides bind to "activated" or angiogenic endothelial cells, and since most endothelium is quiescent, they would not bind to quiescent endothelium, thereby decreasing the potential for background. Tumor-associated endothelium is angiogenic and is therefore recognized by these polypeptides. In addition, since "activation" of endothelium induces the same set of surface markers, any imaging using these polypeptides will not be specific for any particular tumor but rather can be used in general for any angiogenesis-dependent tumor. This is in contrast to imaging agents that target tumor markers, many of which are tumor-type specific.

Suitable detectable labels include radioactive, fluorescent, fluorogenic, chromogenic, or other chemical labels. Useful radiolabels, which are detected simply by gamma counter, scintillation counter, PET scanning or autoradiography include ^{3}H, 124I, ^{125}I, ^{131}I, ^{35}S and ^{14}C. In addition, ^{131}I is a useful therapeutic isotope (see below).

Common fluorescent labels include fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine. The fluorophore, such as the dansyl group, must be excited by light of a particular wavelength to fluoresce. See, for example, Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Sixth Ed., Molecular Probes, Eugene, OR., 1996). Fluorescein, fluorescein derivatives and fluorescein-like molecules such as Oregon Green™ and its derivatives, Rhodamine Green™ and Rhodol Green™, are coupled to amine groups using the isothiocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups. Similarly, fluorophores may also be coupled to thiols using maleimide, iodoacetamide, and aziridine-reactive groups. The long wavelength rhodamines, which are basically Rhodamine Green™ derivatives with substituents on the nitrogens, are among the most photostable fluorescent labeling reagents known. Their spectra are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications. This group includes the tetramethylrhodamines, X-rhodamines and Texas Red™ derivatives. Other preferred fluorophores for derivatizing the peptide according to this invention are those which are excited by ultraviolet light. Examples include cascade blue, coumarin derivatives, naphthalenes (of which dansyl chloride is a member), pyrenes and pyridyloxazole derivatives. Also included as labels are two related inorganic materials that have recently been described: semiconductor nanocrystals, comprising, for example, cadmium sulfate (Bruchez, M. *et al.*, *Science 281*:2013-2016 (1998), and quantum dots, *e.g.*, zinc-sulfide-capped cadmium selenide (Chan, W.C.W. *et al.*, Science 281:2016-2018 (1998)).

In yet another approach, the amino group of a D5 polypeptide is allowed to react with a reagent that yields a fluorescent product, for example, fluorescamine, dialdehydes such as *o*-phthaldialdehyde, naphthalene-2,3-dicarboxylate and anthracene-2,3-dicarboxylate. 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivatives, both chloride and fluoride, are useful to modify amines to yield fluorescent products.

The D5 polypeptides can also be labeled for detection using fluorescence-emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the peptide using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylene-diaminetetraacetic acid (EDTA). DTPA in anhydride form can readily modify the NH_2-containing D5 polypeptides.

For *in vivo* diagnosis or therapy, radionuclides may be bound to the D5 polypeptide either directly or indirectly using a chelating agent such as DTPA and EDTA. Examples of such radionuclides are ^{99}Tc, ^{123}I, ^{125}I, ^{131}I, ^{111}In, ^{97}Ru, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, ^{90}Y and ^{201}Tl. Generally, the amount of labeled D5 polypeptide needed for detectability in diagnostic use will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, contraindications, if any, and other variables, and is to be adjusted by the individual physician or diagnostician. Dosage can vary from 0.01 mg/kg to 100 mg/kg.

The D5 polypeptides can also be made detectable by coupling them to a phosphorescent or a chemiluminescent compound. The presence of the chemiluminescent-tagged peptide is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescers are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the peptides. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

In yet another embodiment, colorimetric detection is used, based on chromogenic compounds which have, or result in, chromophores with high extinction coefficients.

In situ detection of the labeled D5 polypeptide may be accomplished by removing a histological specimen from a subject and examining it by microscopy under appropriate conditions to detect the label. Those of ordinary skill will readily perceive that any of a wide

variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

The term "diagnostically labeled" means that the D5 polypeptide has attached to it a diagnostically detectable label. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes, paramagnetic isotopes, and compounds which can be imaged by positron emission tomography (PET). Those of ordinary skill in the art will know of other suitable labels for binding to the D5 polypeptides used in the invention, or will be able to ascertain such, by routine experimentation.

For diagnostic *in vivo* radioimaging, the type of detection instrument available is a major factor in selecting a radionuclide. The radionuclide chosen must have a type of decay, which is detectable by a particular instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention. Another factor in selecting a radionuclide for *in vivo* diagnosis is that its half-life be long enough so that the label is still detectable at the time of maximum uptake by the target tissue, but short enough so that deleterious irradiation of the host is minimized. In one preferred embodiment, a radionuclide used for *in vivo* imaging does not emit particles, but produces a large number of photons in a 140-200 keV range, which may be readily detected by conventional gamma cameras.

Reagent Compositions

In another embodiment, the D5 polypeptides or derivatives of the present invention are used as affinity ligands for binding to a D5BS in assays, preparative affinity chromatography and solid phase separation of molecules that include a D5BS (such as a D5 receptor). Such compositions may also be used to identify, enrich, purify or isolate cells to which the D55 polypeptide binds, preferably through a specific receptor-ligand interaction using flow cytometric and/or solid phase methodologies. The D5 polypeptide (or derivative) is immobilized using conventional methods, *e.g.* binding to CNBr-activated Sepharose® or Agarose®, NHS-Agarose® or Sepharose®, epoxy-activated Sepharose® or Agarose®, EAH-Sepharose® or Agarose®, streptavidin-Sepharose® or Agarose® in conjunction with biotinylated D5 polypeptide. In general the D5 polypeptides or derivatives of the invention may be immobilized by any other method which is capable of immobilizing these compounds to a solid phase for the indicated purposes. See, for example *Affinity Chromatography: Principles and Methods (Pharmacia LKB Biotechnology)*. Thus, one embodiment is a composition comprising

any of the D5 polypeptides, derivatives or peptidomimetics described herein, bound to a solid support or a resin. The compound may be bound directly or via a spacer, preferably an aliphatic chain having about 2-12 carbon atoms.

By "solid phase" or "solid support" or "carrier" is intended any support or carrier capable of binding the D5 polypeptide or derivative. Well-known supports, or carriers, in addition to Sepharose® or Agarose® described above are glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses such as nitrocellulose, polyacrylamides, polyvinylidene difluoride, other agaroses, and magnetite, including magnetic beads.. The carrier can be totally insoluble or partially soluble. The support material may have any possible structural configuration so long as the coupled molecule is capable of binding to receptor material. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube or microplate well, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, bottom surface of a microplate well, *etc.*

The compositions of the present invention may be used in diagnostic, prognostic or research procedures in conjunction with any appropriate cell, tissue, organ or biological sample of the desired animal species. By the term "biological sample" is intended any fluid or other material derived from the body of a normal or diseased subject, such as blood, serum, plasma, lymph, urine, saliva, tears, cerebrospinal fluid, milk, amniotic fluid, bile, ascites fluid, pus and the like. Also included within the meaning of this term is a organ or tissue extract and a culture fluid in which any cells or tissue preparation from the subject has been incubated.

Pharmaceutical and Therapeutic Compositions and Their Administration

The D5 polypeptides that may be employed in the pharmaceutical compositions of the invention include all of those compounds described above, as well as the pharmaceutically acceptable salts of these compounds. Pharmaceutically acceptable acid addition salts of the compounds of the invention containing a basic group are formed where appropriate with strong or moderately strong, non-toxic, organic or inorganic acids by methods known to the art. Exemplary of the acid addition salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate and nitrate salts.

Pharmaceutically acceptable base addition salts of compounds of the invention containing an acidic group are prepared by known methods from organic and inorganic bases

and include, for example, nontoxic alkali metal and alkaline earth bases, such as calcium, sodium, potassium and ammonium hydroxide; and nontoxic organic bases such as triethylamine, butylamine, piperazine, and tri(hydroxymethyl)methylamine.

As stated above, the D5 polypeptides of the invention possess the ability to inhibit i angiogenesis, properties that are exploited in the treatment of cancer, in particular metastatic cancer. A composition of this invention may be active *per se*, or may act as a "pro-drug" that is converted *in vivo* to the active form.

Compositions within the scope of this invention include all compositions wherein the D5 polypeptide is contained in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 mg/kg/body wt, though more preferred dosages are described for certain particular uses, below.

The D5 polypeptides of the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms, such as capsules, impregnated wafers, tablets or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may be employed.